

# An urokinase receptor antagonist that inhibits cell migration by blocking the formyl peptide receptor

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**Abstract** Urokinase receptor (uPAR) plays a key role in physiological and pathological processes sustained by an altered cell migration. We have developed peptides carrying amino acid substitutions along the Ser<sup>88</sup>-Arg-Ser-Arg-Tyr<sup>92</sup> (SRSRY) uPAR chemotactic sequence. The peptide pyro glutamic acid (pGlu)-Arg-Glu-Arg-Tyr-NH<sub>2</sub> (pERERY-NH<sub>2</sub>) shares the same binding site with SRSRY and competes with *N*-formyl-Met-Leu-Phe (fMLF) for binding to the G-protein-coupled *N*-formyl-peptide receptor (FPR). pERERY-NH<sub>2</sub> is a dose-dependent inhibitor of both SRSRY- and fMLF-directed cell migration, and prevents agonist-induced FPR internalization and fMLF-dependent ERK1/2 phosphorylation. pERERY-NH<sub>2</sub> is a new and potent uPAR inhibitor which may suggest the generation of new pharmacological treatments for pathological conditions involving increased cell migration.

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**Keywords:** Inhibitors of Cell Migration; Formyl-peptide receptor; Urokinase receptor

## 1. Introduction

Cell migration contributes to several diseases driven by aberrant cell motility, such as chronic inflammation, vascular disease and tumor metastasis [1,2]. The receptor for the urokinase-type plasminogen activator (uPAR) plays an important role in physiological processes such as wound healing, inflammation and stem cell mobilization, and in severe pathological conditions such as tumour invasion and metastasis [3]. uPAR is a three domains (D1, D2 and D3) protein anchored to the cell membrane with a glycosyl-phosphatidyl-inositol [4]. uPAR can form a complete signalling unit only when associ-

ated with transmembrane receptors, such as G-protein-coupled receptors [5–8]. The uPAR engagement with uPA favours the exposure of the chemotactic Ser<sup>88</sup>-Arg-Ser-Arg-Tyr<sup>92</sup> sequence which belongs to a flexible linker connecting the uPAR domains D1 and D2 [9,10]. Residues 91–94 are conserved across species [11]. Through Ala-scan, we have found that both Arg residues of the peptide H-Ser-Arg-Ser-Arg-Tyr-OH (SRSRY) are essential to any signalling activity [12]. SRSRY promotes cytoskeletal rearrangements and directional cell migration by binding to the low (FPRL1) or the high *N*-formyl-peptide receptor (FPR) affinity formyl peptide receptors [12–14].

We have developed a series of synthetic peptides carrying specific amino acid substitutions along the SRSRY sequence. In this article, we report that the peptide pyro glutamic acid (pGlu)-Arg-Glu-Arg-Tyr-NH<sub>2</sub> (pERERY-NH<sub>2</sub>) prevents SRSRY chemotactic activity by antagonizing the G-protein-coupled FPR.

## 2. Materials and methods

### 2.1. Peptide synthesis and purification

Peptides were synthesized by the solid phase approach using standard Fmoc methodology in a manual reaction vessel [15]. Peptides were purified by a RP-HPLC C18 column (Vydac 218TP1010) to a 99% purity, as determined by analytical RP-HPLC. Molecular weights were confirmed by mass spectrometry.

### 2.2. Cell cultures

Human Embryonic Kidney HEK-293, Rat Basophilic Leukemia RBL-2H3, and RBL-2H3/ETFR [16] cells were grown in DMEM supplemented with 10% FBS, 100 IU/ml penicillin and 50 µg/ml streptomycin.

### 2.3. Motility assays

Cell migration assays were performed in modified Boyden chambers for 4 h or in Dunn chambers for 6 h as previously described [12,17].

### 2.4. Binding assay

Five micrograms recombinant D2<sub>(88–183)</sub> uPAR fragment (Calbiochem) were biotinylated using a kit purchased from Amersham according to the manufacturer's recommendations. HEK-293 cells (1 × 10<sup>6</sup>) were harvested, incubated with or without the indicated effectors for 15 min at 23 °C and then exposed to biotinylated D2<sub>(88–183)</sub> diluted in binding buffer (DMEM containing 1 mg/ml BSA) for 2 h at 4 °C.

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**Abbreviations:** uPAR, urokinase receptor; pGlu, pyro glutamic acid; fMLF, *N*-formyl-Met-Leu-Phe; FPR, *N*-formyl-peptide receptor

Surface-associated biotinylated proteins were eluted with an acid buffer [18], separated onto a 12.5% SDS-PAGE, and analysed for the biotin content by Western blot.

### 2.5. Fluorescence microscopy

Cells, grown on glass slides to semi-confluence, were incubated with diluents, or the indicated effectors, for 60 min at 4 °C or 30 min at 37 °C. Then, cells were washed with PBS, exposed to *N*-formyl-Nle-Leu-Phe-Nle-Tyr-Lys fluorescein (Molecular Probes) for additional 60 min at 4 °C or 30 min at 37 °C and analysed by a Zeiss LMS510 confocal microscope. Image analysis and quantization of the cell-associated fluorescence were performed on 100 cells, recorded in two different experiments, by using the Axio Vision 4.4 software (Carl Zeiss).

### 2.6. ERK1/2 phosphorylation assay

Cell lysates were prepared with RIPA buffer and cleared by centrifugation as previously reported [12]. Fifty micrograms of proteins/sample were separated on a 10% SDS-PAGE, and transferred to a nitrocellulose membrane. Western blot analysis was performed using 2 µg/ml anti-phospho-ERK1/2 polyclonal antibody. Total ERK1/2 was assessed by re-probing filters with 2 µg/ml anti-ERK1 and anti-ERK2 monoclonal antibodies. Densitometry of autoradiographic bands, was performed by NIH (Bethesda, MD) Image 1.62 software.

### 2.7. Cytosolic $Ca^{2+}$ measurement

Changes in intracellular  $Ca^{2+}$  levels were measured observing with a confocal Zeiss LSM510 microscope (excitation: 488 nm, emission band pass: 500–550) single cells loaded with 5 µM fluo-3AM (Invitrogen-Molecular Probes) for 45 min at 37 °C in humidified air containing 5% CO<sub>2</sub>, and continuously perfused with Krebs solution (160 mM NaCl, 5 mM KCl, 10 mM glucose, 10 mM Hepes, 1.2 mM MgCl<sub>2</sub>, 1.5 mM CaCl<sub>2</sub>, pH 7.4), with or without the peptide under investigation. Offline analysis was performed using the Image J1.37 software (<http://rsb.info.nih.gov/ij/index.html>) and the Time series analyzer plug-in (<http://rsb.info.nih.gov/ij/plugins/time-series.html>).

### 2.8. Statistical analysis

The results were analysed using the Student's *t*-test. A value of  $P < 0.001$  was considered to be significant.

## 3. Results

### 3.1. Selection of pERERY-NH<sub>2</sub> as an antagonist of the SRSRY-directed cell migration

As a first approach, five Glu-scanned SRSRY peptides were synthesized and tested for their ability to promote or to prevent directional cell migration in Boyden chambers. Since all peptides failed to promote HEK-293 cell migration up to 100 nM concentration (not shown), we tested their ability to inhibit SRSRY-dependent cell migration. As expected, HEK-293 cells responded to SRSRY motogen stimulus (280% of the basal migration) [12]. Interestingly, the addition to the lower chamber of equimolar concentrations of H-Ser-Arg-Glu-Arg-Tyr-OH and SRSRY, caused a 31% inhibition of HEK-293 cell migration (Table 1), while the other peptides were ineffective. This finding suggested that the Arg-Glu-Arg central core may be relevant to the inhibition of the SRSRY-dependent cell migration. Therefore, the SRSRY double mutants listed in Fig. 1A were tested for their ability to inhibit SRSRY-dependent HEK-293 cell migration. While the control peptide H-Ala-Arg-Ala-Arg-Tyr-OH (ARARY) did not exert any effect, pGlu-Arg-Glu-Arg-Tyr-OH and H-Glu-Arg-Glu-Arg-Tyr-OH, caused, at 100 pM concentration, a 45% and a 30% inhibition, respectively (Fig. 1A). Furthermore, the C-terminal amidated peptide pGlu-Arg-Glu-Arg-Tyr-NH<sub>2</sub> (pERERY-NH<sub>2</sub>) triggered a 70% inhibition at 100 pM concen-

Table 1

Effects of SRSRY-derived Glu-substituted peptides on SRSRY-directed HEK-293 cell migration

Peptides (10 nM)	Cell migration
None	100 ± 7
H-Glu-Arg-Ser-Arg-Tyr-OH	99 ± 5
H-Ser-Glu-Ser-Arg-Tyr-OH	101 ± 1
H-Ser-Arg-Glu-Arg-Tyr-OH	69 ± 2
H-Ser-Arg-Ser-Glu-Tyr-OH	98 ± 4
H-Ser-Arg-Ser-Arg-Glu-OH	100 ± 8

Chemotactic response of HEK-293 cells to 10 nM SRSRY in a Boyden chamber assay in the presence or in the absence of 10 nM substituted peptides. SRSRY-dependent cell migration (280% of the basal migration) was considered as 100% and cell migration in the presence of substituted peptides was calculated as a percentage of that. Data points are the means ± S.D. of three independent experiments.

tration (Fig. 1A). Further characterization of pERERY-NH<sub>2</sub> shows that this peptide inhibited SRSRY-directed HEK-293 cell migration in a dose-dependent manner. Inhibition starts at 100 fM concentration, 50% of maximal effect being reached at 100 pM. Maximal inhibition was reached in the nM range (Fig. 1B).

### 3.2. pERERY-NH<sub>2</sub> antagonizes FPR signalling

It is known that D2<sub>(88–183)</sub> uPAR fragment binds to FPR or FPRL1 through the SRSRY sequence [12–14]. To assess whether pERERY-NH<sub>2</sub> and SRSRY share the same binding site, HEK-293 cells were pre-incubated with an excess unlabeled D2<sub>(88–183)</sub>, SRSRY, pERERY-NH<sub>2</sub> or ARARY, and then exposed to biotinylated D2<sub>(88–183)</sub>. Eluted proteins were analysed for their biotin content by Western blot. All effectors, with the exception of ARARY, prevented the binding of biotinylated D2<sub>(88–183)</sub> to cell surface to a similar extent, suggesting that both SRSRY and pERERY-NH<sub>2</sub> recognize the same cell surface receptor (Fig. 2A). A likely possibility is that pERERY-NH<sub>2</sub> recognizes FPR which is constitutively expressed by HEK-293 cells [14]. To study the role of FPR, we took advantage of RBL-2H3 cells which are devoid of FPR and RBL-2H3/ETFR which stably express FPR [16]. In a migration assay toward *N*-formyl-Met-Leu-Phe (fMLF), we found that pERERY-NH<sub>2</sub> inhibits RBL-2H3/ETFR cell migration in a dose-dependent manner (Fig. 2B). The inhibitory effect starts at 10 fM concentration, 50% of maximal effect being reached at 1 pM. Maximal inhibition is reached in the low nM range (Fig. 2B). Unlike RBL-2H3 cells, RBL-2H3/ETFR cells move toward SRSRY and fMLF, the extent of cell migration being reduced to the basal levels by 100 pM pERERY-NH<sub>2</sub> (Fig. 2C). In contrast, RBL-2H3 and RBL-2H3/ETFR cell response to fibronectin (Fn) [16] was not prevented by pERERY-NH<sub>2</sub> (Fig. 2C). This indicates that antagonistic effect of pERERY-NH<sub>2</sub> is directed to SRSRY signalling and it is mediated by FPR. To gain some insights into the cellular effects of pERERY-NH<sub>2</sub>, RBL-2H3/ETFR cells were grown adherent onto a glass slide, exposed to a 10 nM fMLF gradient containing 100 pM pERERY-NH<sub>2</sub> or diluents in a DUNN chamber for 6 h, and then stained with rhodamine-phalloidin. Cells subjected to the fMLF gradient exhibited an elongated morphology and recognisable aligned protrusions associated to locomotion in the 80% cell population. Vice versa, the addition of pERERY-NH<sub>2</sub> to the fMLF gradient reduced cell elon-

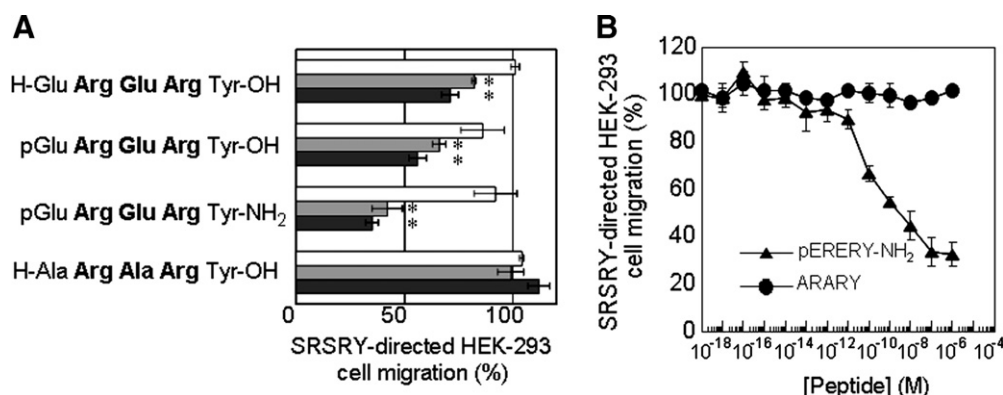


Fig. 1. Screening of peptide inhibitors of the SRSRY-directed cell migration. HEK-293 cells were allowed to migrate in Boyden chambers toward 10 nM SRSRY in combination with 100 fM (□), 100 pM (■), or 100 nM (■) or increasing concentration of the indicated peptides. The extent of cell migration in the presence of the peptides was expressed as a percentage of the SRSRY-dependent directional cell migration assessed in the absence of peptides, considered as 100%. Data represent the means  $\pm$  S.D. of three (A, \* $P$  < 0.0001 compared to SRSRY-dependent directional cell migration) or four (B) independent experiments performed in duplicate.

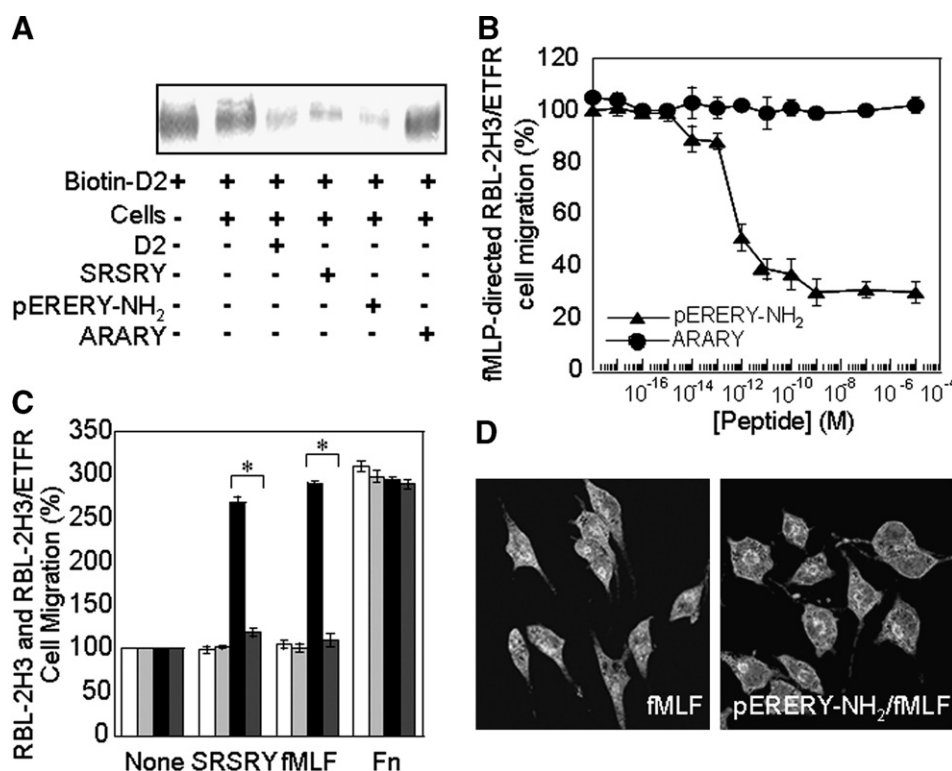


Fig. 2. FPR-dependence of pERERY-NH<sub>2</sub> inhibitory effect. (A) HEK-293 cells were incubated with 500 nM D2<sub>(88–183)</sub>, 500 nM SRSRY, 500 nM pERERY-NH<sub>2</sub>, 500 nM ARARY, or diluents and subsequently exposed to 100 nM biotinylated D2<sub>(88–183)</sub>. Cell surface-associated proteins were eluted and subjected to Western blot analysis. Five microlitres of biotinylated D2<sub>(88–183)</sub> was loaded as a control. (B) Dose-dependent effect of pERERY-NH<sub>2</sub> and ARARY on RBL-2H3/ETFR cell migration directed to 10 nM fMLF. The extent of cell migration was expressed as a percentage of the fMLF-dependent directional cell migration in the absence of peptides (100%). The data represent the means  $\pm$  S.D. of three independent experiments performed in duplicate. (C) Chemotactic response of RBL-2H3 (□, ■), or RBL-2H3/ETFR (■, ■) cells to 10 nM SRSRY, 10 nM fMLF or 50  $\mu$ g/ml fibronectin (Fn), in the presence (■, ■) or in the absence (□, ■) of 100 pM pERERY-NH<sub>2</sub>. The extent of cell migration was expressed as a percentage of the random cell migration assessed in the absence of chemoattractant, considered as 100% (none). The data represent the means  $\pm$  S.D. of independent experiments performed in duplicate (\* $P$  < 0.0001). (D) Representative images of rhodamine-phalloidin stained RBL-2H3/ETFR cells subjected in a DUNN chamber to a 10 nM fMLF chemotactic gradient, in the absence or in the presence of 100 pM pERERY-NH<sub>2</sub>. Original magnification: 100 $\times$ .

gation and alignment with the appearance of F-actin linear distribution along the plasma membranes in at least 75% of cell population (Fig. 2D). Taken together, these data indicate that FPR-mediated pERERY-NH<sub>2</sub> inhibitory effect involves a

marked inhibition of cytoskeletal re-organization occurring during locomotion.

In response to agonist-stimulation, many GPCRs, including FPR, are internalized [19,20]. We first evaluated the direct

binding of pERERY-NH<sub>2</sub> to RBL-2H3/ETFR cells. Cells were pre-incubated at 4 °C with 100 nM fMLF, 100 nM ARARY or increasing concentrations of pERERY-NH<sub>2</sub> and then exposed to *N*-formyl-Nle-Leu-Phe-Nle-Tyr-Lys-fluorescein. As expected, RBL-2H3 cells did not bind to the fluorescent fMLF analog (not shown). However, pERERY-NH<sub>2</sub> specifically inhibited binding of fluorescent agonist to RBL-2H3/ETFR cells in a dose-dependent manner and to a similar extent than fMLF (Fig. 3A). Quantization of the cell-associated fluorescence revealed that, whereas 100 fM pERERY-NH<sub>2</sub> was ineffective, pERERY-NH<sub>2</sub> at 100 pM, 10 nM or 10 μM concentrations caused a 72%, 98% and 94% reduction of ligand binding, respectively. Then, to evaluate the effect of pERERY-NH<sub>2</sub> on agonist-dependent FPR internalization, experiments were performed at 37 °C. Upon exposure to fluorescent agonist, FPR appeared mainly internalized as indicated by punctuate green fluorescent intra-cytoplasmic spots (Fig. 3B) which are undetectable in FPR lacking RBL-2H3

cells (not shown). As expected, intra-cytoplasmic spots were prevented by cell pre-incubation with 100 nM fMLF (Fig. 3B). Unlike ARARY that was ineffective, cell pre-exposure to 100 pM pERERY-NH<sub>2</sub> for 30 min strongly reduced internalization in all cell population (Fig. 3B), suggesting a mechanism in which pERERY-NH<sub>2</sub> inhibits cell migration by preventing the agonist-mediated FPR internalization.

Stimulation of RBL-2H3/ETFR cells with fMLF induces activation of several protein kinases, including MAP kinases [21]. As expected, RBL-2H3/ETFR cell exposure to 10 nM fMLF for 5 or 10 min triggered a time-dependent increase of ERK1/2 phosphorylation. Vice versa, cell exposure to 100 pM pERERY-NH<sub>2</sub> for 5 or 10 min, did not affect the basal ERK1/2 phosphorylation, but consistently decreased the amount of fMLF-induced phosphorylated ERK1/2 (Fig. 3C).

Finally, we assessed whether pERERY-NH<sub>2</sub> interferes with the ability of fMLF to evoke an increase in intracellular

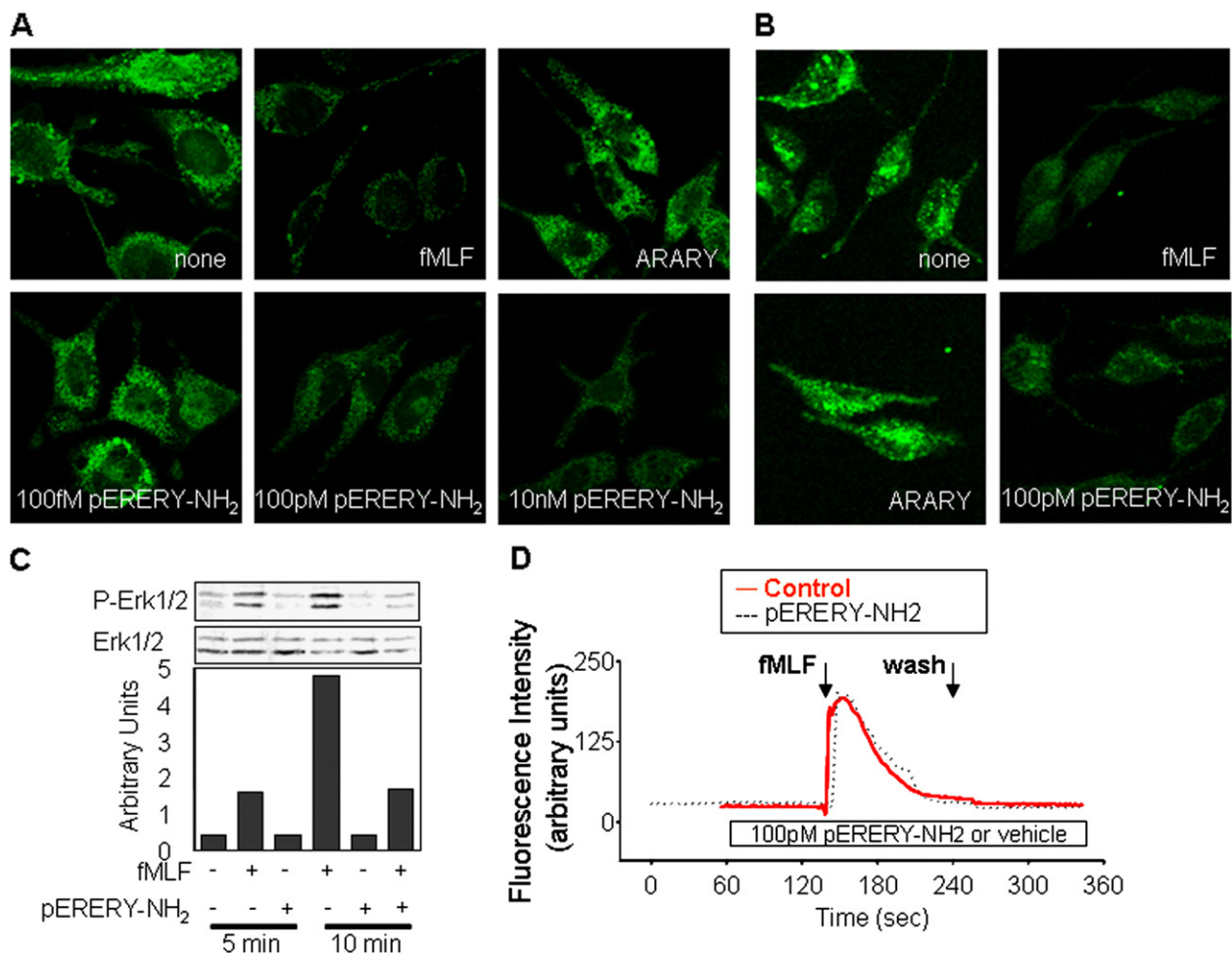


Fig. 3. pERERY-NH<sub>2</sub> inhibits FPR signaling. Representative confocal images of RBL-2H3/ETFR cells incubated at 4 °C (A) or 37 °C (B) with diluents (none), 100 nM fMLF, 100 pM ARARY or pERERY-NH<sub>2</sub> at the indicated concentrations and then exposed to 10 nM *N*-formyl-Nle-Leu-Phe-Nle-Tyr-Lys fluorescein. Original magnification: 630×. (C) Active (P-ERK1/2) and total ERK1/2 levels in RBL-2H3/ETFR cells exposed to 10 nM fMLF or 100 pM pERERY-NH<sub>2</sub> or diluents, or a combination of 100 pM pERERY-NH<sub>2</sub> and 10 nM fMLF for the indicated times. The histogram shows the OD ratio for P-ERK1/2/ERK1/2 bands as quantified by densitometry. A representative experiment of three is shown. (D) Effect of 100 pM pERERY-NH<sub>2</sub> or vehicle on 10 nM fMLF-induced intracellular Ca<sup>2+</sup> response. The panel reports, as superimposed traces, the time course of intracellular Ca<sup>2+</sup> levels, measured as fluo-3 fluorescence intensity, in two different cells, before and after 10 nM fMLF, in the presence of pERERY-NH<sub>2</sub> or vehicle. The traces are representative of 180 cells perfused with vehicle before, during and after the fMLF pulse and 106 cells which after being exposed to Krebs solution during the first minute of recording, were switched to a pERERY-NH<sub>2</sub>-containing solution and kept in the presence of this peptide throughout the entire fMLF stimulation and during its washout.



$\text{Ca}^{2+}$  concentrations. In control cells, fMLF markedly increased intracellular  $\text{Ca}^{2+}$  with a rapid peak (mean percentage increase over baseline  $577.3 \pm 8.2\%$ ,  $n = 180$  in three different experiments) followed by a plateau maintained until the peptide was washed out (Fig. 3D). pERERY-NH<sub>2</sub> neither modified baseline intracellular  $\text{Ca}^{2+}$  (fluo3 fluorescence intensity  $24.3 \pm 1.0$  vs.  $23.9 \pm 1.0$ , ns) nor the intracellular  $\text{Ca}^{2+}$  response evoked by further addition of fMLF (mean percentage increase over baseline  $595.2 \pm 15.7\%$ ;  $n = 106$  in three different experiments) (Fig. 3D). Overall, our findings indicate that pERERY-NH<sub>2</sub> inhibits SRSRY function by antagonizing FPR-dependent MAP-K activation without interfering with the effects of this receptor on intracellular  $\text{Ca}^{2+}$  homeostasis.

#### 4. Discussion

We have developed synthetic peptides carrying specific amino acid substitutions along the SRSRY uPAR sequence. Among these, the peptide H-Ser-Arg-Glu-Arg-Tyr-OH was selected for its ability to inhibit SRSRY-directed cell migration. Further modifications led to the generation of the peptide pGlu-Arg-Glu-Arg-Tyr-NH<sub>2</sub> (pERERY-NH<sub>2</sub>), which is a potent inhibitor of both SRSRY- and fMLF-directed cell migration. In an effort to characterize the molecular mechanisms underlying the activity of pERERY-NH<sub>2</sub>, we demonstrated that pERERY-NH<sub>2</sub> shares the same binding site with SRSRY on the FPR and prevents: (a) binding of a fluorescent fMLF analogue to RBL-2H3/ETFR cell surfaces; (b) fMLF-directed cell migration of RBL-2H3/ETFR cells stably expressing FPR; (c) agonist-dependent FPR internalization and ERK1/2 phosphorylation. These remarkable effects are due to its binding to the FPR.

Many reports clearly show the involvement of the uPAR in cell migration, thus suggesting that this receptor may be regarded as a new molecular target for therapy of diseases sustained by increased cell motility. In this respect, efforts have been made to interfere with the uPA/uPAR or integrin/uPAR interactions [9,21,22]. To the best of our knowledge, pERERY-NH<sub>2</sub> is the first uPAR inhibitor that specifically impairs the uPAR/FPR interaction.

Furthermore, pERERY-NH<sub>2</sub> can be regarded also as a true FPR antagonist. A large body of literature shows that FPRs govern a variety of cellular functions, including chemotaxis which requires ERK1/2 phosphorylation, but not a cytosolic  $\text{Ca}^{2+}$  increase [23]. In our experiments, pERERY-NH<sub>2</sub> blocks FPR-mediated chemotaxis and ERK1/2 phosphorylation, without affecting intracellular calcium mobilization. Since we have not detected any cell response following exposure to pERERY-NH<sub>2</sub> alone, it is possible that this peptide might behave as a neutral antagonist. pERERY-NH<sub>2</sub> appeared to recognize FPR with a higher affinity (pM) than fMLF or SRSRY (nM) which instead promote chemotaxis. In this respect, pERERY-NH<sub>2</sub> could act as an inverse agonist by shifting, upon binding to FPR, the active state of the receptor toward the inactive one. Regarding the specific affinity of pERERY-NH<sub>2</sub> for its target, competition experiments with a fluorescent fMLF analogue showed that a concentration as low as 100 pM pERERY-NH<sub>2</sub> prevents both internalization and ligand-uptake of FPR. Labelled pERERY-NH<sub>2</sub> is unavailable to us, and therefore we could not determine the affinity of pERERY-NH<sub>2</sub> for the FPR. How pERERY-NH<sub>2</sub> interacts with

FPR and what structural basis dictates its high affinity for FPR remain to be investigated. Also, it would be interesting to determine whether pERERY-NH<sub>2</sub> behaves as a neutral antagonist or an inverse agonist for FPR. This issue is relevant in vivo for potential therapeutic applications, since inverse agonist effects are associated with receptor activation and inactivation, whereas neutral antagonism produces no effect when administered alone, but blocks the effects of agonists and inverse agonists [19].

pERERY-NH<sub>2</sub> is a novel FPR antagonist exhibiting a higher affinity as compared with other antagonists of FPR that have been investigated for their putative role as therapeutic agents [24,25]. In particular, the cyclic undecapeptide, cyclosporine H is a potent and selective FPR inverse agonist which prevents both FPR-mediated  $\text{Ca}^{2+}$  mobilization and chemotaxis [24]. pERERY-NH<sub>2</sub> behaves differently from cyclosporine H because it does not prevent FPR-mediated  $\text{Ca}^{2+}$  mobilization and it is much more potent than cyclosporine H in inhibiting fMLF-induced chemotaxis ( $\text{IC}_{50} = 1$  pM and 49 nM, respectively).

GPCRs have a crucial role in the physiopathology of immune system as well as in cancer progression and metastasis. In particular, accumulating evidence suggests that modulating GPCR function might delay the progression of many cancers and their spread to distant organs [26]. Given the finding that pERERY-NH<sub>2</sub> inhibits cell migration by antagonizing FPR, it is reasonable to foresee that this peptide may be employed to develop new drugs for the treatment of diseases that are sustained by a chronic excess of cell migration, such as inflammatory diseases, tumor spread and metastases.

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